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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BEJ A K ET AL: "DETECTION OF COLIFORM BACTERIA AND ESCHERICHIA COLI BY MULTIPLEX POLYMERASE CHAIN REACTION: COMPARISON WITH DEFINED SUBSTRATE AND PLATING METHODS FOR WATER QUALITY MONITORING" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 57, no. 8, August 1991 (1991-08), pages 2429-2432, XP001004829 ISSN: 0099-2240 abstract page 2429 --- -/--	25, 26

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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(57) Abstract: The present invention pertains to methods, reagents, compositions, kits and instruments for use in simultaneously amplifying multiple targets. In particular, this invention is based on the discovery of a two-step multiplex amplification reaction wherein the first step truncates the standard initial multiplex amplification round to "boost" the sample copy number by only a 100 - 1000 fold increase in the target. Following the first step the product is divided into optimized secondary single amplification reactions, each containing one of the primer sets that were used previously in the first or multiplexed booster step. The booster step can occur using an aqueous target nucleic acid or using a solid phase archived nucleic acid.

METHODS AND DEVICES FOR MULTIPLEXING AMPLIFICATION REACTIONS

BACKGROUND OF THE INVENTION

1. Field of the Invention:

5 The present invention pertains to methods, reagents, compositions, and diagnostic kits, for use in simultaneously amplifying multiple nucleic acid targets. In particular, this invention is based on the discovery of a two-step multiplex amplification reaction wherein the first step truncates a standard multiplex amplification round to thereby "boost" the sample copy number by
10 only a 100 - 1000 fold increase in the target. Following the first step of the present invention, the resulting product is divided into optimized secondary single amplification reactions, each containing one of the primer sets that were used previously in the first or multiplexed booster step. The booster step can occur using an aqueous target nucleic acid or using a solid phase
15 archived nucleic acid.

2. Description of the State of the Art:

Genetic information is stored in living cells in threadlike molecules of DNA. *In vivo*, the DNA molecule is a double helix, each strand of which is a chain of nucleotides. Each nucleotide is characterized by one of four bases:
20 adenine (A), guanine (G), thymine (T), and cytosine (C). The bases are complementary in the sense that due to the orientation of functional groups, certain base pairs attract and bond to each other through hydrogen bonding. Adenine in one strand of DNA pairs with thymine in an opposing complementary strand and guanine in one strand of DNA pairs with cytosine
25 in an opposing complementary strand. In RNA, the thymine base is replaced by uracil (U) which pairs with adenine in an opposing complementary strand.

Another distinction between DNA and RNA is the nature of the pentose sugar each contains. Specifically, DNA consists of covalently linked chains of deoxyribonucleotides and RNA consists of covalently linked chains of
30 ribonucleotides.

Each nucleic acid is linked by a phosphodiester bridge between the five prime hydroxyl group of the sugar of one nucleotide and the three prime hydroxyl group of the sugar of an adjacent nucleotide. Each linear strand of naturally occurring DNA or RNA has one terminal end having a free five prime hydroxyl group (referred to as the 5' end) and another terminal end having a three prime hydroxyl group (referred to as the 3' end). Complementary strands of DNA and RNA form antiparallel complexes in which the three prime terminal end of one strand is oriented to the five prime terminal end of the opposing strand.

10 Nucleic acid hybridization assays are based on the tendency of two nucleic acid strands to pair at complementary regions. Presently, nucleic acid hybridization assays are primarily used to detect and identify unique DNA and RNA base sequences or specific genes in a complete DNA molecule in mixtures of nucleic acid, or in mixtures of nucleic acid fragments.

15 Since all biological organisms or specimens contain nucleic acids of specific and defined sequences, a universal strategy for nucleic acid detection has extremely broad applications in a number of diverse research and development areas as well as commercial industries. The identification of unique DNA or RNA sequences or specific genes within the total DNA or RNA extracted from tissue or culture samples may indicate the presence of physiological or pathological conditions. In particular, the identification of unique DNA or RNA sequences or specific genes, within the total DNA or RNA extracted from human or animal tissue, may indicate the presence of genetic diseases or conditions such as sickle cell anemia, tissue compatibility, cancer and precancerous states, or bacterial or viral infections. The identification of unique DNA or RNA sequences or specific genes within the total DNA or RNA extracted from bacterial cultures or tissue containing bacteria may indicate the presence of antibiotic resistance, toxins, viruses, or plasmids, or provide identification between types of bacteria.

30 The potential for practical uses of nucleic acid detection was greatly enhanced by the description of methods to amplify or copy, with fidelity,

precise sequences of nucleic acid found at low concentration to much higher copy numbers, so that they are more readily observed by detection methods.

The original amplification method is the polymerase chain reaction described by Mullis, *et al.*, in his U.S. Patent Nos. 4,683,195; 4,683,202 and 5 4,965,188; all of which are specifically incorporated herein by reference. Subsequent to the introduction of PCR, a wide array of strategies for amplification has been described. See, for example, United States Patent No. 5,130,238 to Malek, entitled "Nucleic Acid Sequence Based Amplification (NASBA)"; United States Patent No. 5,354,668 to Auerbach, entitled 10 "Isothermal Methodology"; United States Patent No. 5,427,930 to Buirkenmeyer, entitled "Ligase Chain Reaction"; and, United States Patent No. 5,455,166 to Walker, Entitled "Strand Displacement Amplification (SDA)"; all of which are specifically incorporated herein by reference.

In general, diagnosis and screening for specific nucleic acids using 15 nucleic acid amplification techniques has been limited by the necessity of amplifying a single target sequence at a time. In instances where any of multiple possible nucleic acid sequences may be present, performing multiple separate assays by this procedure is cumbersome and time consuming. For example, the same clinical symptoms generally occur due to infection from 20 many etiological agents and therefore requires differential diagnosis among numerous possible target organisms. Cancer prognosis and genetic risk is known to be due to multiple gene alterations. Genetic polymorphism and mutations result from alterations at multiple loci and further demand determination of zygosity. In many circumstances the quantity of the targeted 25 nucleic acid is limited so that dividing the specimen and using separate repeat analyses is often not possible. There is a substantial need for methods enabling the simultaneous analysis of multiple gene targets for the same specimen. For amplification-based methods this has been called "multiplex reactions".

30 Chamberlain, *et al.*, *Nucleic Acid Research*, 16:11141-11156 (1988) first demonstrated multiplex analysis for the human dystrophin gene. Specific

primer sets for additional genetic diseases or infectious agents have subsequently been identified. See, Caskey, *et al.*, European Patent No. EP 364,255A3; Caskey, *et al.*, United States Patent No. 5,582,989, Wu, *et al.*, United States Patent 5,612,473 (1997). The strategy for these multiplex
5 reactions was accomplished by careful selection and optimization of specific primers. Developing robust, sensitive and specific multiplex reactions have demanded a number of specific design considerations and empiric optimizations. See, Edwards and Gibbs, *PCR Methods Applic.*, 3:S65-S75 (1994); Henegariu, *et al.*, *Biotechniques*, 23:504-511 (1997). This results in
10 long development times and compromises reaction conditions that reduce assay sensitivity. Because each multiplex assay requires restrictive primer design parameters and empirical determination of unique reaction conditions, development of new diagnostic tests is very costly.

A number of specific problems have been identified that limit multiplex
15 reactions. Incorporating primer sets for more than one target requires careful matching of the reaction efficiencies. If one primer amplifies its target with even slightly better efficiency, amplification becomes biased toward the more efficiently amplified target resulting in inefficient amplification of other target genes in the multiplex reaction. This is called "preferential amplification" and
20 results in variable sensitivity and possible total failure of one or more of the targets in the multiplex reaction. Preferential amplification can sometimes be corrected by carefully matching all primer sequences to similar lengths and GC content and optimizing the primer concentrations, for example by increasing the primer concentration of the less efficient targets. One
25 approach to correct preferential amplification is to incorporate inosine into primers in an attempt to adjust the primer amplification efficiencies (Wu, *et al.*, United States Patent 5,738,995 (1998). Another approach is to design chimeric primers. Each primer contains a 3' region complementary to sequence-specific target recognition and a 5' region made up of a universal
30 sequence. Using the universal sequence primer permits the amplification efficiencies of the different targets to be normalized. See, Shuber, *et al.*, *Genome Research*, 5:488-493 (1995); Shuber, United States Patent

5,882,856. Chimeric primers have also been utilized to multiplex isothermal strand displacement amplification (Walker, *et al.*, United States Patents 5,422,252, 5,624,825, and 5,736,365).

Since multiple primer sets are present, multiplexing is frequently
5 complicated by artifacts resulting from cross-reactivity of the primers. In an attempt to avoid this, primer sequences are aligned using computer BLAST or primer design programs. All possible combinations must be analyzed so that as the number of targets increases this becomes extremely complex and severely limits primer selection. Even carefully designed primer combinations
10 often produce spurious products that result in either false negative or false positive results. The reaction kinetics and efficiency is altered when more than one reaction is occurring simultaneously. Each multiplexed reaction for each different specimen type must be optimized for $MgCl_2$ concentration and ratio to the deoxynucleotide concentration, KCL concentration, Taq
15 polymerase concentration, thermal cycling extension and annealing times, and annealing temperatures. There is competition for the reagents in multiplex reactions so that all of the reactions plateau earlier. As a consequence, multiplexed reactions in general are less sensitive than the corresponding simplex reaction.

20 Another consideration to simultaneous amplification reactions is that there must be a method for the discrimination and detection of each of the targets. Generally, this is accomplished by designing the amplified product size to be different for each target and using gel electrophoresis to discriminate these. Alternatively, probes or the PCR products can be labeled
25 so as to be detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, using multiple fluorescent dyes incorporated with a self-quenching probe design amplification can be monitored in real time. See, Livak, *et al.*, United States Patent 5,538,848 (1996); Livak, *et al.*, United States Patent 5,723,591 (1998);
30 and Di Cesare, United States Patent 5,716,784. The number of multiplexed targets is further limited by the number of dye or other label moieties distinguishable within the reaction. As the number of different fluorescent

moieties to be detected increases, so does the complexity of the optical system and data analysis programs necessary for result interpretation. Another approach is to hybridize the amplified multiplex products to a solid phase then detect each target. This can utilize a planar hybridization platform with a defined pattern of capture probes (Granados, *et al.*, United States Patent 5,955,268 (1999), or capture onto a beadset that can be sorted by flow cytometry (Chandler, *et al.*, United States Patent 5,981,180 (1999)).

Due to the summation of all of the technical issues discussed, current technology for multiplex gene detection is costly and severely limited in the number and combinations of genes that can be analyzed. Generally, the reactions multiplex only two or three targets with a maximum of around ten targets. Isothermal amplification reactions are more complex than PCR and even more difficult to multiplex. See, Van Deursen, *et al.*, *Nucleic Acid Research*, 27:e15 (1999).

There is still a need, therefore, for a method which permits multiplexing of large numbers of targets without extensive design and optimization constraints. There is also a further need for a method of detecting a significantly larger number of gene targets from a small quantity of initial target nucleic acid.

SUMMARY OF THE INVENTION

Accordingly, it is a general object of this invention to provide a method that permits the multiplex amplification of multiple targets without extensive design and optimization constraints.

Another object of the invention is to provide a method that enables amplification of multiple targets (or multiple amplification assays) from limited quantity specimens with very low nucleic acid copy number.

A further object of the invention is to provide a diagnostic kit that allows the user to perform amplification of multiple targets without extensive design and optimization constraints and to amplify targets from limited quantity specimens with very low nucleic acid copy number.

Additional objects, advantages and novel features of this invention shall be set forth in part in the description that follows, and in part will become apparent to those skilled in the art upon examination of the following specification or may be learned by the practice of the invention. The objects
5 and advantages of the invention may be realized and attained by means of the instrumentalities, combinations, compositions, and methods particularly pointed out in the appended claims.

To achieve the foregoing and other objects and in accordance with the purposes of the present invention, as embodied and broadly described
10 therein, the method of this invention comprises a two-step multiplex amplification reaction wherein the first step truncates a standard multiplex amplification round thereby resulting in a product having a boosted target copy number while minimizing primer artifacts. The second step divides the resulting product achieved in the first step into optimized secondary single
15 amplification reactions, each containing one of the primer sets that were used previously in the first step.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form a part of the specification, illustrate the preferred embodiments of the present
20 invention, and together with the description serves to explain the principles of the invention.

In the Drawings:

Figure 1 is a diagrammatic illustration of the limited multiplex amplification method of the present invention.

25 Figure 2a is an illustration of an agarose gel showing the results of the experiment in Examples 1 and 2.

Figure 2b is an illustration of an agarose gel showing the results of the experiment in Examples 1 and 2.

30 Figure 2c is an illustration of an agarose gel showing the results of the experiment in Examples 1 and 2.

Figure 2d is an illustration of an agarose gel showing the results of the experiment in Examples 1 and 2.

Figure 3 is an illustration of an agarose gel showing the results of the experiment in Example 1-3.

5 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

As discussed above, it would be tremendously useful if a method and diagnostic kit could be devised to multiplex multiple nucleic acid targets without the necessity of complex design constraints and extensive optimizations. The methods and diagnostic kit of the present invention
10 generally involve the use of common multiplex amplification methods and reagents and are more specifically derived from the surprising discovery that if the nucleic acid sample to be analyzed is first pre-amplified so as to merely "boost" the samples copy number slightly, then the resulting product may be split into as many subsequent analysis reactions as required, and thereby
15 circumventing the common limitations of standard multiplexing technology.

The present invention describes a novel method for boosting the amount of nucleic acid obtained from a broad range of biological samples for distribution among large numbers of individual analyses. It preferably involves the utilization of archived nucleic acid, bound irreversibly to a solid-
20 phase material as the starting material. See U.S. application for patent, Serial No. 09/061,757, and corresponding international application WO 98/46797 which are both incorporated herein by reference. The copy number of the irreversibly bound nucleic acid is then "boosted" by running a limited multiplex amplification reaction. The limited multiplex amplification reaction is a
25 truncated version of any well known amplification methods such as, but not limited to PCR, RT-PCR, NASBA, SDA, TMA, CRCA, Ligase Chain Reaction, etc. Using archived nucleic acid has the advantage that multiple sequential rounds of the present invention may be performed. Alternatively, nucleic acids that are not bound but in aqueous solution may also be used. In this
30 instance nucleic acid is obtained from the desired biological sample by a number of common procedures. These include phenol-chloroform and/or

ethanol precipitation (Maniatis, *et al.*, *Molecular Cloning; A Laboratory Manual*), high Salt precipitation (Dykes, *Electrophoresis* 9:359-368 (1989) chex and other boiling methods (Walsh, *et al.*, *Biotechniques*, 10:506-513 (1991) and other solid phase binding and elution (Vogelstein and Gillespie, 5 *Proc. Nat. Acad. Sci. USA*, 76:615-619 (1979) etc. Output from these initial rounds of limited amplifications are distributed into the several single analyses.

In the preferred embodiment of the present invention, a sample containing tissue, cells or other biological material is treated in the presence 10 of the solid phase binding material to release the nucleic acid contained in that sample. The solid phase archiving material allows amplification without elution of the bound nucleic acid. Once the sample nucleic acid is bound to the solid phase, the solid phase material is washed to remove lysis buffer conditions, and to prepare for amplification conditions. A reaction mixture 15 appropriate to the amplification method is added to the solid phase or allowed to contact the solid phase. The number of primer pairs used according to the present invention may be any number greater than two; however, since the standard multiplexing reaction conditions and designs become more difficult and less effective as the number of primers used increases the present 20 invention is most helpful as the number of primers utilized is over five.

This reaction mixture contains the amplification primers for several independent amplifications. This is similar to standard multiplex PCR with the following exceptions: first, in the preferred embodiment, the primer concentrations will be very low. The effective primer concentrations should be 25 limited to allow only a few logs of amplification but definitely the primer concentration should be exhausted before reaction plateau is reached. Second, the number of amplification cycles should also be minimized. The goal of this first phase of multiplex amplification or "Booster Amp" is to allow only a sufficient amount of amplification to proceed in order to allow the 30 resultant reaction mix to be split up and redistributed into at least the same number of subsequent simple amplification reactions as there are independent primer pairs in the first amplification.

This initial round of amplification, or Booster Amp should only proceed into early logarithmic phase of the amplification, and in no instance proceed until reaction plateau is reached. Once the Booster Amp is complete, the resultant reaction mix with the multiple amplification species is removed from contact with the solid phase and distributed into several secondary amplifications. These amplifications could be equal to or greater than the number of primer pairs employed in the first Booster Amp. Each of these secondary amplification reactions will contain only one primer pair. This primer pair may be identical to one of the primer pairs in the Booster Amp or may be "nested" within one of the primer pairs of the Booster Amp. Either way, each secondary amplification reaction will use the input material from the Booster amplification as a target source. In the secondary amplification, normal amounts of primer will be used, and amplification will be allowed to proceed normally. A detection system for normal detection of a single amplification product such as, but not limited to radioactive isotopes, or visual markers such as biotin may be included in the secondary amplification.

In the preferred embodiment the reaction takes place in the presence of a solid phase material as discussed previously. The advantage of this is that the original solid phase material with the bound nucleic acid may be rinsed following the first Booster Amp and re-initialized for a second, subsequent Booster Amp employing a new mix of amplification primers. The process is then repeated through an additional secondary amplification. The entire process can be repeated for numerous rounds. Consequently, in the event the quantity of nucleic acid sample being analyzed is low, the analysis may be performed as often and as thoroughly as need be. Alternatively, the Booster Amp step may be performed in aqueous condition where the nucleic acid is unbound.

Figure 1 illustrates the concept for microwell based PCR (but can apply to any primer-based amplification method utilizing a polymerase, such as but not limited to DNA polymerase, RNA polymerase, transcriptase, or Q β replicase in any form of an appropriate container). A chip or card containing the reaction wells or chambers (not shown) is contained in a device capable

of performing the correct thermocycling. Lysed sample is introduced into extraction chamber 10, where the freed nucleic acid can bind to the solid phase material within extraction chamber 10. The chamber 10 having bound the sample is incubated in step 12 for a short period (10-20 minutes). An aqueous buffer, preferably PCR buffer is then used in washing step 14. The wash 14 removes lysate and initializes the chamber 10' in step 16 for PCR conditions. The first multiplex PCR reaction mixture (containing multiplexed primers, PCR buffer, and Taq Polymerase) is introduced to the chamber 10 and cycled in step 18. The multiplex products 20' should be sub-detectable, but amplified to a level no greater than the plateau of the reaction and preferably in the range of 100 to 1000-fold. The Booster PCR reaction 20' is then split into the secondary PCR chambers 22'. Reaction mixtures (having a single primer pair) for each of the simplex, secondary PCR reactions (not shown) are introduced previously, or at this point. Cycling is performed on the chip or card to allow the secondary PCR reactions 22' to proceed to completion. The initial sample chamber is now empty, and it can be washed at step 24 and re-initialized for a second round of Booster/Secondary PCRs.

This invention reveals a robust and simple method for multiplex amplification of large numbers of gene targets. The invention teaches that preferably 2-40 or more primer sets can be combined to permit multiplex amplification if they are in low concentration and limited to an initial amplification round that results in only a 100-1000 fold increase in target. However, it should be understood that any number of primer sets greater than two may be used according to the present invention. This has been designated as a "booster round" or booster amp and can occur using an aqueous target nucleic acid or using solid phase archived nucleic acid. As discussed above, the advantage of using archived material is that multiple booster rounds can be performed from the same archived specimen. For example, performing five, 20-target booster rounds from archived nucleic acid would permit the analysis of 100 different genes. Following each booster round the amplification product is diluted into optimized secondary single PCR reactions, each containing one of the primer sets that were multiplexed in the

booster reaction. These simplex reactions can be optimized for maximum sensitivity and each requires only one method of detection, for example single dye homogeneous detection. The invention enables multiplexing without extensive optimization and is robust enough to permit random selection of the primers to be multiplexed.

The invention overcomes the usual technical problems of multiplexing. By limiting the multiplexed cycles, preferential amplification and cross-reaction of the multiple primers is minimized. Only the single PCR reactions need to be optimized. The simplex reaction has maximum sensitivity since reagent competition does not occur. By using the simplex PCR the detection probe does not have to be multiplexed. The potential to randomly combine the multiplexed primers provides for maximum flexibility and cost effectiveness since this allows custom selection of the targets to be multiplexed. Frequently, the targets that need to be multiplexed can vary for a particular geographic location, laboratory, type of patient, or type of specimen. Since archived nucleic acid can be reanalyzed the multiplex can be designed in a logical algorithm. For example, in booster reaction detection set one, identify the most frequently known mutations. Then only if these are not detected is it necessary to perform additional multiplexes for the more rare mutations. This enables economical yet comprehensive genetic analysis.

The invention is further illustrated by the following non-limited examples. All scientific and technical terms have the meanings as understood by one of ordinary skill in the art. The specific examples which follow illustrate the various multiplexing amplification methods that the present invention may be adapted to work with and are not to be construed as limiting the invention in sphere or scope. The methods may be adapted to variation in order to function with other commonly used multiplex amplification methods embraced by this invention but not specifically disclosed. Further, variations to the methods to produce the same results in somewhat different fashion will be evident to one skilled in the art.

All temperatures are understood to be in degrees Centigrade (°C) when

not specified. Melting temperatures (T_m) of the primers were estimated using the generally accepted mathematical calculation based upon the formula $T_m = 81.5 + 16.6 \times \log (Na^+) (41 \times (\#G + \#C)/\text{length}) - 500/\text{length}$. Amplification techniques, including multiplexing amplification techniques are now

5 sufficiently well known and widespread so as to be considered routine. All polymerase enzymes and nucleotides can be purchased from PE (Biosystems, Foster City, California). PCR was carried out in a buffer containing (50mM KC1, 10 mM Tris, pH 8.3, 2.0mM Mg^{2+}) for 30 cycles of 1 minute at 94°C, for 2 minutes at 55°C and at 72°C with a 5 second increment

10 added to the 72°C elongation step at every cycle. This procedure was carried out in a DNA Thermal Cycler #N8010150 (Perkin-Elmer Cetus).

Example 1

Multiplex Amplification Incorporating Booster PCR and Archiving.

1. Primer Design:

15 The Xtra Amp™ Extraction Kit (Xtrana, Inc.) provides an innovative system for nucleic acid extraction in which the nucleic acid remains bound in the extraction tube and can be directly amplified by PCR in this same tube. The unique principle underlying this system lies in the proprietary nucleic acid binding matrix, Xtra Bind™. Xtra Bind™ is a non-silica matrix which stably

20 and irreversibly binds both DNA and RNA. The Xtra Amp™ kit contains 96 (as 1 X 8 strips) 0.2ml size microcentrifuge tubes coated with the Xtra Bind™ matrix, cell lysis buffer and wash buffer. The kit is commercially available currently for extraction of genomic DNA from, whole blood (manufactured and distributed for Xtrana, Inc. by ANSYS Diagnostics, Lake Forest, California).

25 For demonstrating Xtra Plex feasibility, the Xtra Amp Blood kit was chosen as the extraction platform. For the PCR multiplexing experiments it was decided to obtain twenty five primer pairs to allow their use in various groupings. Three primer sets from the Lifecodes Corporation (Stanford, Connecticut) HLA Primers (HLA-A: Primer A1 (cat# 164011); Primer A2 (cat# 164012);

30 HLA-B: Primer B1 (cat#165011), Primer B2 (cat# 165012; DRβ: Primer A (cat# 160031); Primer B (cat# 160032) were added to twenty three in house

primer sets, shown below in Table 1, that were designed for human gene targets to make the total of twenty five sets. The genes targeted are as follows: Human cytoplasmic beta-actin gene, (accession M10277); Homo sapiens interleukin 2 precursor (IL2) gene, (accession J00264); Human growth hormone gene (HGH-N), (accession M13438); Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, (accession J04038); Homo sapiens dystrophin (DMD) gene (accession AF214530); Homo sapiens G protein-coupled receptor 57 (GPR57) gene, (accession AF112461); Human galactokinase (GALK1) gene, (accession L76927); Homo sapiens MHC class 1 region. (accession AF055066). The primer pairs were designed using the primer design software OLIGO 5.0 (Molecular Biology Insights). The sizes of the amplification products are designed to allow discrimination by agarose gel electrophoresis. No special considerations were given to normalizing the melting temperatures of the primer pairs, or to eliminating structural interactions between primers from different pairs. PCR primer design considerations such as avoiding hairpins and dimers between the forward and reverse primers of each set were accommodated in the design as for any typical PCR primer design. The resultant primers had melting temperatures ranging from 49° C to 72° C. When examining the dimer formation potential between primers from different pairs, some serious dimer potential was seen in some cases. This was largely ignored since this approach was believed to be able to overcome such potential artifacts and it was considered important to demonstrate this. The primers and sequences are shown in Table 1.

25

TABLE I

Primer Pair	Group-ings	Sequence	Name	TM	Amplicon Length
	Grp. 1				
1	1	CGAGGCCCGAGAGCAA	HBAPCR1-FP	58	100
	2	GGTGTGGTGCCAGATTT	HBAPCR1-RP	57	100
2	3	GTTTAATCAGAGCCACA	IL2 =PCR2-FP	52	187
	4	GGAAAGGTAGGTCAAGA	IL2 PCR2-RP	54	187
3	5	GTCTTGCAATTGCACTAA	IL2-OLD-fp	52	257
	6	TAAATGTGAGCATCCTG	IL2-OLD-rp	52	257
4	7	CTGTGGAGGGCAG CTGT	hGH PCR1-FP	68	450
		GGCTT			

	8	GCGGGCGGATTACCTGA	hGH PCR1-RP	68	450
		GGTCA			
5		Lifecodes HLA-A, A1	HLA-A-A1	N/A	910
		Lifecodes HLA-A, A2	HLA-A-A2	N/A	910
	Grp. 2				
6	9	TCAGCAGAGAAGCCTAA	IL2-PCR1-FP	54	120
	10	ATCCCTACCCCATCAT	IL2-PCR1-RP	54	120
7	11	CAAAAGTCCTCCCAAAG	IL2-PCR3-FP	54	197
	12	TGCCATCTATCACAATCC	IL2-PCR3-RP	55	197
8	13	AAGGGTCATCATCTCTGC	GAPDH I5 fp	57	259
	14	CTTCCACGATACCAAAGTT	GAPDH I5 rp	55	259
9	15	CGCTTTAAGTTATTTGTGTC	HDYST3-FP	54	400
	16	GTTTCCTTTTAAGGGTATTC	HDYST3-RP	54	400
10		Lifecodes HLA-B, B1	HLA-B -B1	N/A	1100
		Lifecodes HLA-B, B2	HLA-B -B1	N/A	1100
	Grp. 3				
11	17	CATCTACGAGGGGTATG	HBAPCR2-FP	57	120
	18	GCCGTGGTGGTGA	HBAPCR2-RP	54	120
12	19	GTTTGCCTTTTATGGTAATAAC	HBAPCR4-FP	55	161
	20	GTGAGCTGCGAGAA	HBAPCR4-RP	54	161
13	21	GAGTCCACTGGCGTCTTCAC	GAPDH FP	64	233
	22	AGGCTGTTGTCATACTTCTC	GAPDH RP	58	233
14	23	CCACCCCTTAAAGAAA	IL2-PCR4-FP	54	346
	24	GGCAGGAGTTGAGGTTA	IL2-PCR4-RP	57	346
15	25	GCGGGGAGGAGGAAAGGAATAG	hGHPCR2-FP	66	500
	26	CAGGACACATTGTGCCAAAGGG	hGHPCR2-RP	64	500
	Grp. 4				
16	27	CCACTATTCGGAACTT	HGP57R1-FP	52	130
	28	TGTATGGCATAATGACA	HGP57R1-RP	49	130
17	29	GAGTCGAGGGATGGCTAGGT	HDYST1-FP	64	150
	30	TTCAAAGTGGGATGAGGAGG	HDYST1-RP	60	150
18	31	GGACTGCCACCTTCTACC	HGKPCR2-FP	62	215
	32	GACACCCAAGCATACACC	HGKPCR2-RP	59	215
19	33	GCAGATGAGCATACGCTGAGTG	hGHPCR3-FP	64	600
	34	CGAGGGGAAATGAAGAATACGG	hGHPCR3-RP	62	600
20		Lifecodes DR-β, A	DR-β, A	N/A	287
		Lifecodes DR-β, B	DR-β, B	N/A	287
	Grp. 5				
21	35	AGGGGAGGTGATAGCAT	HBAPCR3-FP	57	140
	36	AAGTTGGGGGACAAAA	HBAPCR3-RP	51	140
22	37	CCGGTGCCATCTTCATA	HGKPCR1-FP	68	170
	38	CCTGCCTTGCCCATTCTT	HGKPCR1-RP	68	170
23	39	GAGGGGAGAGGGGGTAA	HBAPCR5-FP	62	228
	40	CGGCGGGTGTGGA	HBAPCR5-RP	57	228
24	41	GGCTGCTTTTAACTCTGG	GAPDH FP	57	270
	42	CACCTCTGGAAGATGGTGATGG	GAPDH RP	64	270
25	43	CTCATTCTCTAGCCAAATCT	HDYST2-FP	56	300
	44	CCTCGACTCACTCTCCTC	HDYST2-RP	62	300
	New Primers				
26	45	CTATCGCCATCTAAGCCCAGTA	HGH PCR4-fp	62	450
	46	CTGCCTGCATTTTCACTTCA	HGH PCR4-Rp	58	450

2. Sequential Booster Reaction of Solid-Phase Captured Nucleic Acid.

In these multiplexing experiments, (the results of which are demonstrated in Figures 2a-2d) designed to show feasibility for performing multiple rounds of multiplexing amplification, each booster amplification consisted of a PCR reaction with five primer sets. In the first 5-plex experiment, Group 2 primers were used. In the second experiment group 4 primers were used, and in the third, group 1 primers were used. In the 20 plex experiment, the primers used were selected from all 5 groups each at one-fiftieth normal concentration (4 nM as opposed to 200 nM). The nucleotide triphosphate, buffer and salt concentrations were normal. Cycling conditions were chosen as a compromise between the theoretically optimal conditions for each of the primer pairs (each cycle: 72° C, 30 sec; 55° C, 1 min; and 65° C, 3 min). The total number of cycles was limited to ten. The primers were first mixed in 1:1 ratios to each and then diluted to one-fiftieth of the normal 10x concentration. The PCR reaction mix for the booster reaction was made up as per normal. The Xtra Amp kit (discussed previously) was used with fresh human blood as per the kit protocol. The booster reactions were placed into the Xtra Amp tubes as would a normal PCR. Following the booster PCR, the reaction mixture was removed and diluted 5-fold.

To each simplex, secondary reaction 5 microliters of this diluted booster product was added. Each secondary reaction had only one of the 5 primer pairs and was set up normally with normal amounts of primer. These PCRs were run for the full 40 cycles. Two cycling profiles (72°C, 30 sec; 65° C, or 1 min; 72°C, 3 min) were used for these secondary reactions. The same cycling profile used in the booster reactions was used for all secondary PCRs. In cases where the primers were believed to require higher temperatures, additional secondary reactions were run using higher temperature profiles (each cycle: 72°C, 30 sec; 65°C, 1 min; 72°C, 3 min).

Next, the Xtra Amp tube in which the booster reaction was performed was rinsed three times with Wash Buffer supplied in the kit. A second booster PCR reaction mixture with a different set of five primer pairs was added to the

tube. Identical conditions were used as in the first booster amplification. The product was removed and diluted as before, and aliquoted into the next group of five secondary, simplex PCRs using the same primer pairs as in the second booster PCR. All of these secondary reactions were run using the same
5 cycling profile as the booster, with some primers being additionally run at the higher temp profile as well. Following this, a third round of booster/secondary simplex reactions were run with a third grouping of five primers in identical conditions to the first two rounds.

For comparison, a normal, 40-cycle multiplex PCR was run using each
10 of the five primer pair groupings. Each of these had five primer pairs each in the same groupings as the booster PCRs. These multiplex reactions were run with half-normal primer concentration for 40 cycles. The products from all fifteen secondary reactions, the high-temp additional secondary reactions and the three normal multiplexing PCRs were analyzed by agarose gel
15 electrophoresis.

Figure 2a demonstrates the results of the first round of the secondary PCR reactions. Five PCRs were used in first round (Group 2: IL2-1; IL2-3; GAPDH I5; HDYST3; HLA-B) booster PCR. The Booster PCR was performed in an Xtra Amp tube following extraction of nucleic acids from human blood.
20 Following booster amplification, product was diluted 1:5, then 1:10 volume of this was added to six subsequent reactions in separate normal PCR tubes. The six subsequent simplex secondary reactions included all five of the Booster PCR systems, with an additional system (HLA-A) whose primers were similar to one of the five (HLA-B). The additional PCR served as a
25 secondary PCR control and was expected to be negative. The results shown in Figures 2a demonstrate that all five PCRs from Booster worked in the secondary simplex reactions. In contrast, a normal multiplex PCR (MMX) in which all five primer pairs were run in a similarly prepared Xtra Amp tube for a total of 40 cycles did not work for all PCR products. (In this and all gel images
30 pictured, the reference ladder is in 100 base-pair increments starting at 100 bp L is ladder, nt is no template and t is template).

Figure 2b demonstrates the results of the second round of the secondary PCR reaction. The second round of 5-primer pair booster PCR was run in the same Xtra Amp tube as was the first booster. The tube was washed three times with Xtra Amp kit wash buffer prior to the addition of the second booster multiplex PCR mix. The next five primer pairs were from Group 4: HGP57; HDYST-1; HGK-2; HGH-3, and DRB. Following an identically run booster PCR, the product was diluted 1:5 as before and aliquoted into the five secondary simplex PCR reactions. These reactions were run at the low temperature cycling range. The results shown in Figure 2b demonstrates that the four reactions worked at this temperature with some spot contamination in one of the HDYST-1 no-template controls. The HGP57 and HGH-3 secondary reactions work best at the high temperature secondary cycling protocol and were done at that temperature as well. A normal PCR multiplex (MMX) with these same primers in an Xtra Amp tube failed again.

Figure 2c demonstrates the results of the third round of the secondary PCR reactions. In this experiment, a third 5-primer pair booster multiplex was performed in the same Xtra Amp tube as was the first two booster reactions, discussed above, using primer sets from group 1 (HBA PCR 1; IL2 PCR 2; IL2 PCR-old; hGH PCR 1 and HLA-A). Again, three standard washes were done with the tube between the previous booster PCR and this one. The standard 40-cycle multiplex with these primers failed with these primers as well. The results shown in Figure 2c demonstrates that some contamination in the no template controls was seen in the IL2-OLD primer reaction, and the hGH-1 PCR did not work well with the low temperature cycling condition set for the secondary reactions. Both the hGH-1 and HLA-A secondary reactions were run at the high temperature secondary cycling condition set (65° C and 72° C vs. 55° C and 65° C).

The results shown in Figure 2d demonstrates that in this set of secondary reactions, the primer sets which appeared to be compromised in the first sets of secondary reactions were rerun using higher annealing and extension temperatures (65° C and 72° C vs. 55° C and 65° C respectively). The contamination issue with IL2-OLD was still apparent (this primer set had

been used previously in our lab). The remaining PCR reactions were much more robust.

The results indicate that all of the secondary reactions worked. In one case where a primer pair had been used extensively in this lab prior to these experiments (IL2-OLD) there appeared some contamination in the no template controls. One primer pair gave weak positive results (HGP-57). Four primer pairs worked better at the higher temperature secondary PCR profile. One primer pair worked better at higher concentration in the secondary reaction. These results indicate that the secondary PCR reactions function as normal PCR reactions in which some optimization will give better results. The booster PCR was able to function adequately at less than optimal conditions for the various primer sets. In contrast, the three normal multiplex PCRs that were run as multiplex amplifications for the full 40 cycles failed to show amplification products for all but a few primer sets.

3. Multiplex Capability Enabled by Booster PCR:

Since the two-step PCR approach in which a limited-booster PCR is used to boost the target copy prior to aliquoting the boosted product into the individual secondary PCRs worked so well with groups of 5 primer pairs, increasing the number of primer pairs in the booster reaction was performed. A 20-primer pair booster reaction was set up using identical conditions as before, except that in addition to using the Xtra Amp kit with human blood as the sample, a booster PCR was set up in a normal PCR tube using previously extracted human placental DNA as the template. The primer set 23 replaced primer set 4 in this experiment. The individual primers were at 4 nM or one-fiftieth of normal concentration. The booster PCR was run for 10 cycles using the lower temperature profile (each cycle: 72°C, 30 sec; 55°C, 1 min; 65°C, 3 min). The product for this booster reaction was diluted 5-fold and used as template for the 20 secondary, simplex PCR reactions. The products from these reactions were analyzed by agarose gel electrophoresis as shown in Figure 3. Although the contrast of the pictures obscures the results in some cases, all reactions gave product at the expected sizes for the aqueous

PCRs. In some instances the aqueous reactions gave slightly better results than was seen for the reactions whose booster PCR was done in Xtra Amp tubes. Further optimization of the secondary reactions should eliminate those differences. The last four reactions (HGH-3, HLA-A, HLA-B and DR were
5 done using the high temperature cycling condition set in the secondary reactions).

The results showed that all of the secondary PCRs from the aqueous (non-Xtra Amp) booster worked. Some of the secondary PCRs still need some minor optimization in order to yield cleaner products, but correctly sized
10 product bands were seen for all reactions. Three secondary reactions from the Xtra Amp booster showed very weak product (poorly reproduced on gel photo). Additional optimization of the secondary reactions should improve the performance. It is noteworthy that even with 40 different primers being present in the booster PCRs, the secondary reactions worked yielding
15 products of the correct size. This indicates that primer-primer artifacts across primer systems that may form in the booster reaction, and would most certainly form in a traditional one-step multiplex, do not interfere significantly with the secondary simplex reactions.

By performing the two-step multiplex of multiplex booster followed by
20 simplex secondary reactions, large numbers of assays can be done with a single sample without encountering the confounds associated with normal single-step multiplex amplification. Of further note is the fact that even using PCR primers that have not been optimized in any way, and boosting with a profile that is demonstrably less than optimal for at least some of the primer
25 sets used, the approach is still robust.

Example 2

Sequential Booster Xtra Plex NASBA Reaction of Solid-Phase Captured Nucleic Acid

1. Booster Xtra Plex NASBA First Pass

30 Several NASBA systems are currently in operation in our lab. From these ten primer sets, shown in Table 2 below, were chosen. These were: For

Escherichia coli :SZ gene (SZ); SLT1 & 2 genes (SLT1, SLT2); 16S ribosomal RNA (16S), LacZ –(LacZ 1 and LacZ 2); and UIDA gene (UIDA), for *Listeria monocytogenes* (HlyA gene); for *Neisseria gonorrhea* 16s ribosomal RNA-(NG2), and for *Chlamydia trachomatis* 16s ribosomal RNA(CT2). Surprisingly, the primers LACZ 1 and LACZ 2 have been shown to be specific to *E. coli* and can discriminate *E. coli* from other coliform species. The 16s ribosomal (16s) primer set is capable of binding to and allowing amplification of all gram-negative bacteria examined and may be a universal primer set for all gram-negative bacteria. The primer sets LACZ 1, LACZ 2 and 16S (see sequences) are *E. coli*-specific primers and gram-negative universal primers, respectively.

Table 2

Organism	Seq. ID. #	Gene	Sequence
<i>Chlamydia trachomatis</i>	47	CT2:FP	AATTCTAATACGACTCACTATAGGGAGAGGTAACCG AAAGGTCCTAAGAT
	48	CT2 RP	ATTGTTTAGTGGCGGAAGGG
	49	CT2 5DP	FITC-ACTTGGGAATAACGGTTGGAAA-PO ₄
	50	CT2 3DP	GCTAATACCGAATGTGGCGATA-Biotin
<i>Neisseria gonorrhea</i>	51	NG2 FP	AATTCTAATACGACTCACTATAGGGAGAACTG ACATCGTTTAGGGCGTGG
	52	NG2 RP	AATGCGTAGAGATGTGGAGG
	53	NG 5DP	FITC-AGCCTCCTGGGATAAACTGACG-PO ₄
	54	NG 3DP	AGCGTGGGTAGCAAACAGGATTAGA-Biotin
Coliform Bacteria	55	LacZ 1 FP	AATTCTAATACGACTCACTATAGGGAGAGG AAACTGCTGCTGGTGTGTTTGCTT
	56	LacZ 1 RP	TTACGGCGGTGATTTTGGCGAT
	57	LacZ 1 5DP	FITC-ATCGCCAGTTCTGTATGA-PO ₄
	58	LacZ 1 3DP	CCGACCGCACGCCGCATCCAGC-Biotin
Coliform Bacteria	59	LacZ 2FP	ATTCTAATACGACTCACTATAGGGAGAGGAGAA CTGGAAAACTGCTGCTGG
	60	LacZ 2 RP	CGTTTACAGGGCGGCTTCGTCT
	61	LacZ 5DP	FITC-ATCGCCAGTTCTGTATGA-PO ₄
	62	LacZ 3DP	CCGACCGCACGCCGCATCCAGC –Biotin
Coliform Bacteria	63	UIDA FP	AATTCTAATACGACTCACTATAGGGAGA GGAATAGTCTGCCAGTTCAGTTCGTTGT
	64	UIDA RP	CAAAGTGTGGGTCAATAATCAGGAA
	65	UIDA 5DP	FITC-CTATACGCCATTTGAAGCCGAT-PO ₄
	66	UIDA 3DP	GTCACGCCGTATGTTATTGCCG-Biotin
<i>Escherichia coli</i> O157:H7	67	SZ FP	TTGTTAGCGTTACGTTTCCCTCT
O157:H7	68	SZ RP	AATTCTAATACGACTCACTATAGGGAGAG GATAATACCAAATCAGGTTTTCCATTGA

	69	SZ 5DP	FITC-CGATGATGCTACCCCTGAAAACT-PO ₄
	70	SZ 3DP	GAGAATGAAATAGAAGTCGTTGTT-Biotin
<i>Escherichia coli</i> O157:H7	71	SLT 1 FP	GTTTGCAGTTGATGTCAGAGG
O157:H7	72	SLT 1RP	ATTCTAATACGACTCACTATAGGGAGAGGAA CGTGGTATAGCTACTGTC
	73	SL 1 5DP	FITC-ATCTACGGCTTATTGTTGAACGAAA-PO
	74	SL 1 3DP	TTTTATCGCTTTGCTGATTTTTCAC - Biotin
<i>Escherichia coli</i>	75	SLT 2 FP	TTGCTGTGGATATACGAGGG
	76	SLT 2 RP	ATTCTAATACGACTCACTATAGGGAGAG GAGAGTGGTATAACTGCTGTC
	77	SLT 2 5DP	FITC-TTTTGACCATCTTCGTCTGATTATT-PO ₄
	78	SLT 3 DP	GTTAATACGGCAACAAATACTTTCT -Biotin
<i>Escherichia coli</i>	79	16S FP	AATTCTAATACGACTCACTATAGGGAGAGGACC TTGTTACGACTTCACCCAG
	80	16S RP	TACACACCGCCCGTCACACCAT
<i>Listeria monocytogenes</i>	81	HLYA FP	AATTCTAATACGACTCACTATAGGGAGAACCT TTTCTTGGCGGCACA
	82	HLYA RP	GTCCTAAGACGCCAATCGAA
	83	HL 5DP	FITC-AACACGCTGA TGAAATCTATAAGTATA-PO ₄
	84	HL 3DP	GTATTAGTATACCACGGAGATGCAGTG-Biotin

PRIMER AND PROBE SEQUENCES FOR NASBA: FP = FORWARD PRIMER; RP = REVERSE PRIMER; 5DP = 5'-LATERAL FLOW DETECTION PROBE; and 3DP = 3'-LATERAL FLOW DETECTION PROBE

5 Various cell extracts and artificial RNA templates were pooled (extracted RNAs, RNA runoff material, and RNA product from previous NASBA reactions). Twenty microliters of each were combined then diluted 1:1 with water. Thirty microliters of this mix was added to Xtra Amp tubes. To this was then added an equal volume of 2X LiCl lysis buffer. The solution was
10 mixed and let sit for 20 minutes at room temperature. After 3 washes with LiCl wash buffer, the tubes were ready for NASBA.

Two sequential booster groups of 5 primer pairs each were then run in tandem. The booster NASBA reactions were run with normal reaction components and concentrations except that the primers were pooled and run
15 at one-fiftieth of normal concentration (4 nM instead of 200 nM). Two booster reactions were set up in parallel and run for different times. The first was run for 15 minutes at 40°C, and the second for 30 minutes at 40°C. The reactions

were terminated and the products removed from the tubes. The tubes were washed for the next round. The products were diluted 10-fold and used as template for the subsequent secondary simplex reactions. These reactions were run normally and analyzed by agarose gel electrophoresis and lateral
5 flow where possible. The second group of five primer sets was then run identically in the same Xtra Amp tubes as were used in the first group booster. The procedure from booster to secondary simplex amplifications was identical. The groupings of the primer pairs were as follows: Group 1: SZ; SLT2; HlyA; CT2; and LacZ2; Group 2: SLT1; 16S; NG2; LacZ1 and UIDA1.

10 The results indicated that four of the five secondary reactions from the first booster reaction worked as indicated either by agarose gel electrophoresis or lateral flow detection. The system that failed, *E. coli* SLT-2, is a system that has a history of inconsistent performance in our laboratory. All five of the secondary reactions from the second booster reaction worked.
15 The gel results and lateral flow (Gerdes, U.S. Patent 5,989,813 incorporated herein by references) results varied slightly. This variance has been seen with these systems previously and is indicative of sub-optimal conditions. Thus, although the reaction conditions require further optimization for full performance in the Booster Xtra Plex paradigm, the results are extremely
20 encouraging and noteworthy in that they were so positive with completely untested and highly preliminary conditions.

2. Multiplex Capability Enabled by Booster NASBA:

Multiplexing isothermal amplification reactions is even more difficult than multiplexing PCR reactions. However there are applications in which an
25 isothermal amplification of multiple targets would be the method of choice if that were possible. By employing the booster strategy developed for PCR but with NASBA primers this has been accomplished. A short booster NASBA reaction in which the ten primer pairs targeting genes products from diverse organisms was performed as above, but with all ten present in the same
30 reaction. The booster NASBA was run with the HlyA NASBA condition set using 1:50 diluted primer mix per set (4.0 nM final concentration per primer).

Template materials (extracted RNAs, RNA runoff material, and RNA product from previous NASBA reactions) for each primer set were pooled and diluted and used as template for the booster reaction. The template material was bound onto the Xtra Bind material in an Xtra Amp tube as above. This

5 reaction was run at 40°C for 15 minutes and 30 minutes. The products from these reactions were diluted ten-fold and used as template for the ten separate, secondary simplex NASBA reactions.

The results of the sequential booster Xtra Plex NASBA reaction of solid-phase captured nucleic acid, shown in Table 3 below, indicate that eight

10 of the ten NASBA systems worked. One of the failures was the same system as in the above experiment. Again, the results are noteworthy in that at the time of this writing, even multiplexing two distinct primer sets in a NASBA reaction has not been demonstrated.

Table 3

15 Sequential Booster Xtra Plex NASBA Reaction of Solid-Phase Captured Nucleic Acid Results

Primer Pair	Agarose Gel Results	Lateral Flow Results
Group 1:		
<i>E. coli</i> SZ	Negative	Positive
<i>E. coli</i> SLT 2	Negative	N/A
<i>L. monocytogenes</i> HlyA	Positive	Positive
<i>C. trachomatis</i> 16s	Positive	Positive
<i>E. coli</i> LacZ 2	Positive	Positive
Group 2:		
<i>E. coli</i> SLT 1	Negative	Positive
<i>E. coli</i> 16s	Positive	N/A
<i>N. gonorrhea</i> 16s	Positive	Positive
<i>E. coli</i> LacZ 1	Positive	Negative
<i>E. coli</i> UIDA	Positive	Negative
10 Primer Pair NASBA Boosted Simplex Reactions		
<i>E. coli</i> SZ	Negative	Negative
<i>E. coli</i> SLT 2	Negative	N/A
<i>L. monocytogenes</i> HlyA	Positive	Positive
<i>C. trachomatis</i> 16s	Positive	Positive
<i>E. coli</i> LacZ 2	Positive	Positive
<i>E. coli</i> SLT 1	Negative	Positive
<i>E. coli</i> 16s	Positive	N/A
<i>N. gonorrhea</i> 16s	Positive	Positive
<i>E. coli</i> LacZ 1	Positive	Negative
<i>E. coli</i> UIDA	Positive	Negative

The foregoing description is considered as illustrative only of the principles of the invention. The words "comprise," "comprising," "include," "including," and "includes" when used in this specification and in the following claims are intended to specify the presence of one or more stated features, integers, components, or steps, but they do not preclude the presence or addition of one or more other features, integers, components, steps, or groups thereof. Furthermore, since a number of modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and process described above. Accordingly, all suitable modifications and equivalents may be resorted to falling within the scope of the invention as defined by the claims which follow.

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CLAIMS

What is claimed is:

1. A method for simultaneously amplifying multiple nucleic acid sequence targets contained in a sample of single stranded nucleic acid by an
5 amplification method comprising:
 - (a) contacting the sample with at least two primer pairs;
 - (b) pre-amplifying the sample for a period of time less than necessary to achieve reaction plateau;
 - (c) dividing said pre-amplified sample into at least two separate
10 vessels; and
 - (d) adding at least one of said primer pairs to said amplified sample and amplifying said divided sample.
2. The method of claim 1, wherein said sample is DNA.
3. The method of claim 1, wherein said sample is RNA.
- 15 4. The method of claim 1, wherein said pre-amplifying step is accomplished using polymerase chain reaction.
5. The method of claim, wherein said amplifying step is accomplished using polymerase chain reaction.
6. The method of claim 1, wherein said pre-amplifying step is
20 accomplished using isothermal methodology.
7. The method of claim 1, wherein said amplifying step is accomplished using isothermal methodology.
8. The method of claim 1, wherein said pre-amplifying step is accomplished using nucleic acid sequence based amplification.
- 25 9. The method of claim 1, wherein said amplifying step is accomplished using nucleic acid sequence based amplification.

10. The method of claim 1, wherein said pre-amplifying step is accomplished using nucleic acid sequence based amplification using ligase chain reaction.

11. The method of claim 1, wherein said amplifying step is
5 accomplished using nucleic acid sequence based amplification using ligase chain reaction.

12. The method of claim 1, wherein said pre-amplifying step is accomplished using nucleic acid sequence based amplification strand displacement amplification.

10 13. The method of claim 1, wherein said amplifying step is accomplished using nucleic acid sequence based amplification.

14. The method of claim 1, wherein 15-40 primer pairs are contacted with said sample.

15 15. The method of claim 1, wherein said pre-amplification results in only a 100 to 1000 fold increase in the targets

16. The method of claim 1, wherein said pre-amplified sample is divided into a number portions that equal the number of primer pairs used.

17. The method of claim 1, wherein the sample is irreversibly bound to a high affinity solid phase matrix.

20 18. The method of claim 1, wherein the sample is unbound in an aqueous environment

19. A method for simultaneously amplifying multiple single stranded nucleic acid targets, comprising:

- (a) contacting at least two primer pairs with the single stranded
25 nucleic acid targets;
- (b) pre-amplifying said targets 100 – 1000 fold;
- (c) dividing said pre-amplified targets into separate reaction vessels

wherein the number of said vessels used is equal to the number of primer pairs;

(d) amplifying said pre-amplified targets.

20. The method of claim 19, wherein the target is amplified with a
5 polymerase.

21. The method of claim 20, wherein the target is amplified with a polymerase. in a DNA polymerase, an RNA polymerase, a transcriptase, or Q β replicase.

22. The method of claim 20, wherein the single stranded nucleic
10 acid targets are DNA polynucleotides and the polymerase is a DNA polymerase.

23. The method of claim 19, wherein said targets are in an aqueous solution.

24. The method of claim 19, wherein said targets are irreversibly
15 bound to a solid phase matrix.

25. A method for discriminately identifying *E. coli* from other coliform species, comprising:

- (a) contacting the primer sets Seq ID. Nos. 55, 56, 57, 58, 59, 60,
61, and/or 62 with a sample target;
20 (b) amplifying said target; and
(c) detecting said amplified target.

26. A method for discriminately identifying *E. coli* from other coliform species, comprising:

- (a) contracting the primer sets Seq ID. Nos. 79 and 80 with a
25 sample target;
(b) amplifying said target; and
(c) detecting said amplified target.

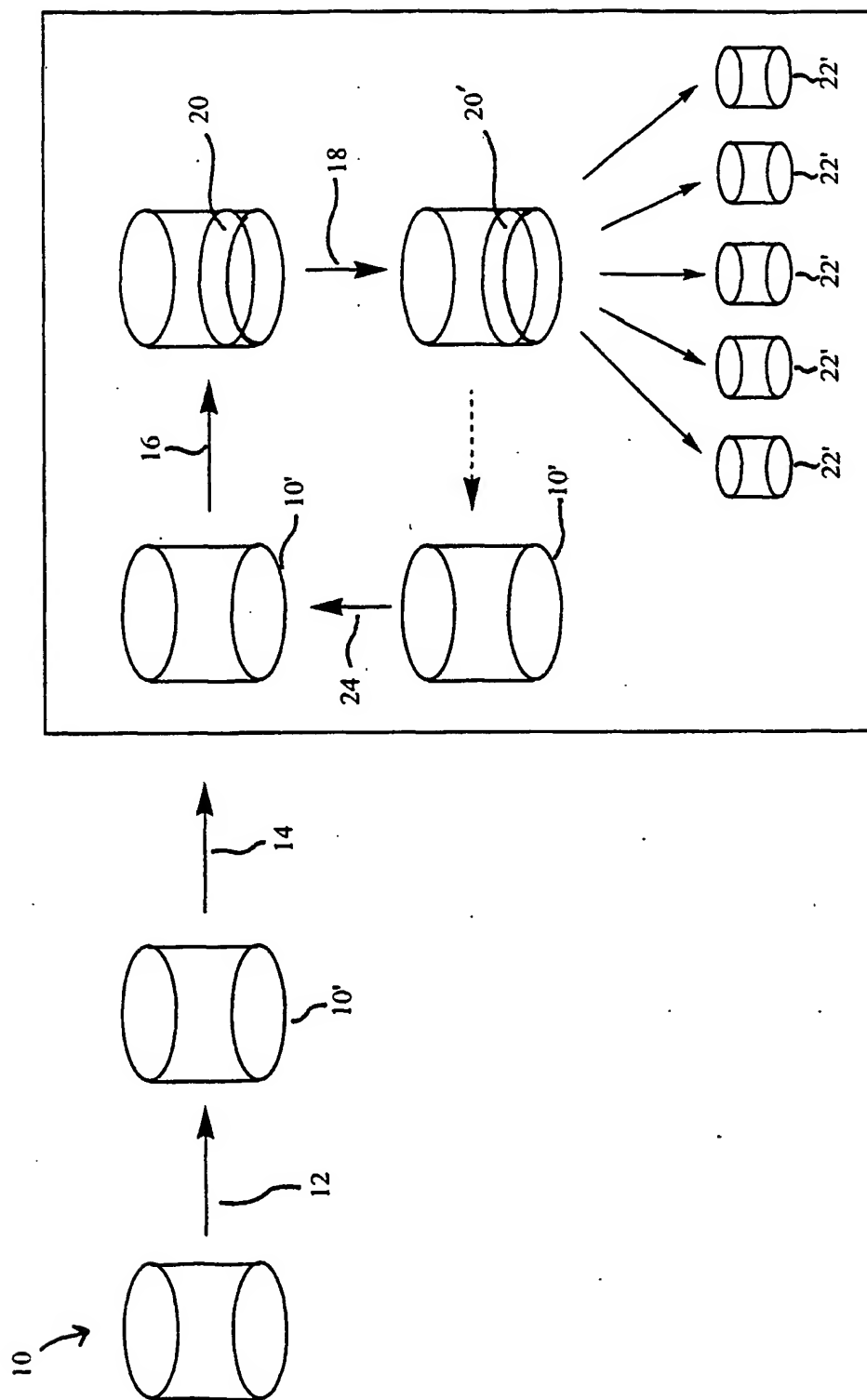


Figure 1

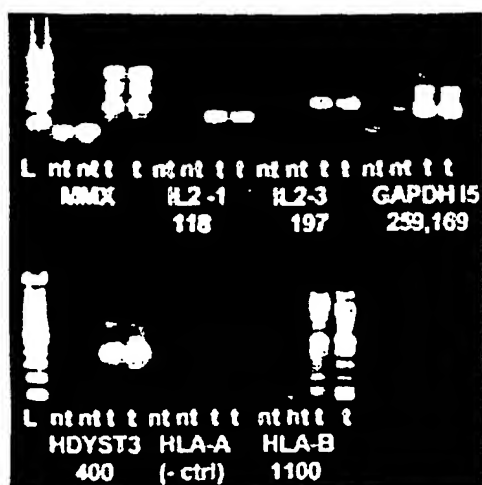


Figure 2A

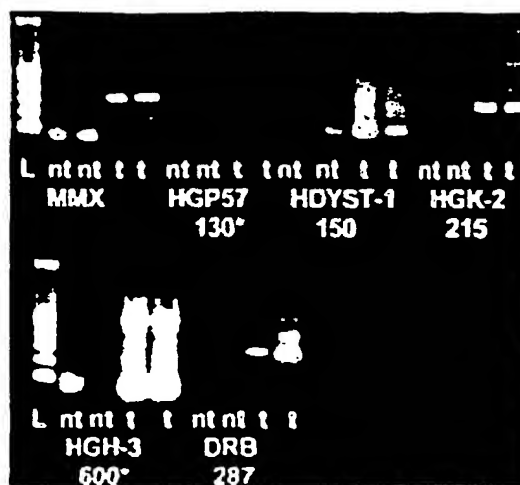


Figure 2B

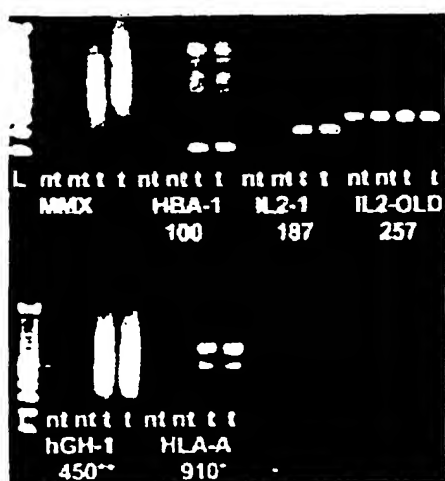


Figure 2C



Figure 2D

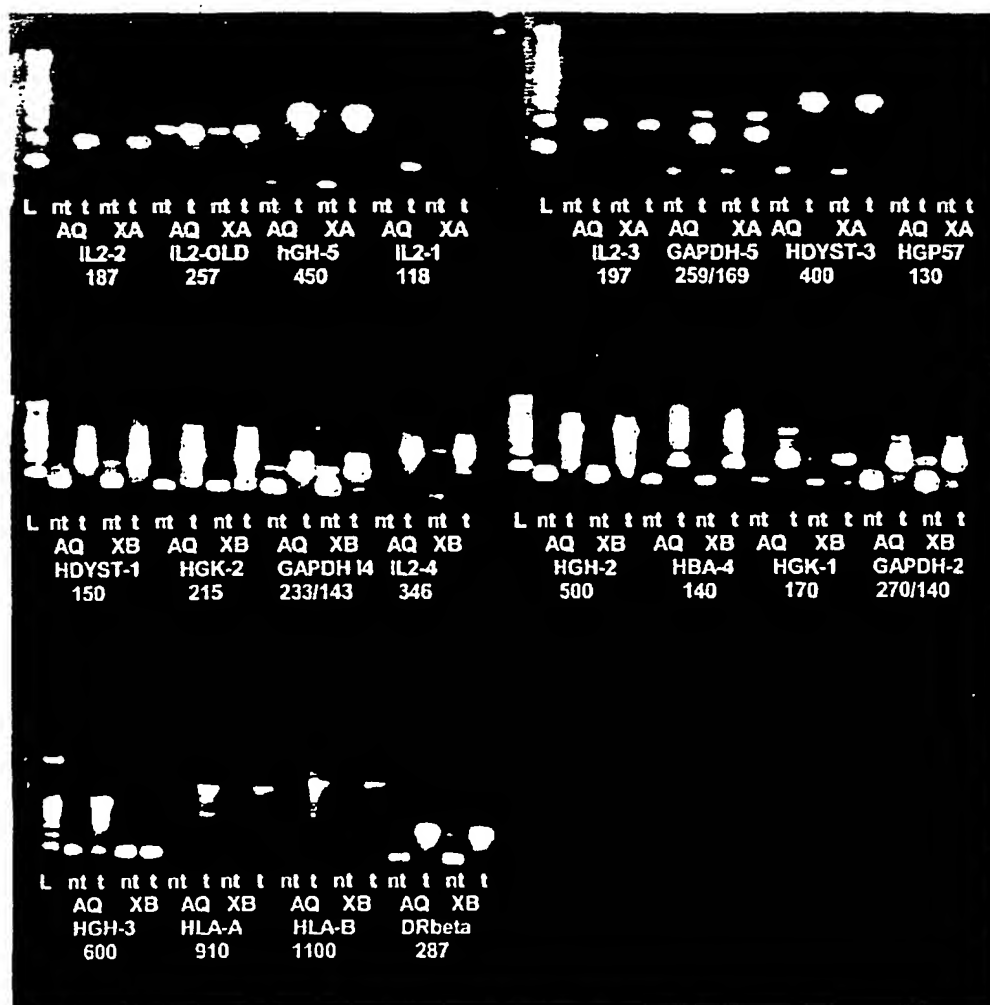


Figure 3.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	<p>EDWARDS M C ET AL: "Multiplex PCR: advantages, development, and applications." PCR METHODS AND APPLICATIONS. UNITED STATES FEB 1994, vol. 3, no. 4, February 1994 (1994-02), pages S65-S75, XP009003160 ISSN: 1054-9803 cited in the application</p>	25,26
Y	<p>the whole document, in particular: p.568, left column, 1st and 2nd full paragraphs; p.569, 1st and 3rd full paragraphs; p.571, 3rd full paragraph.</p>	1-24
Y	<p>WO 97 43441 A (DUNN JAMES M ;LEUSHNER JAMES (CA); SHIPMAN ROBERT (CA); VISIBLE GE) 20 November 1997 (1997-11-20) abstract page 2, line 30 -page 5, line 26 page 7, line 23 -page 8, line 13</p> <p>---</p>	1-24
Y	<p>GONIN P ET AL: "Performance of a multiplex PCR for the determination of Haemophilus influenzae capsular types in the clinical microbiology laboratory." DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE. UNITED STATES MAY 2000, vol. 37, no. 1, May 2000 (2000-05), pages 1-4, XP002225665 ISSN: 0732-8893 abstract page 2, left-hand column, paragraph 2 -page 3, left-hand column, paragraph 2</p> <p>---</p>	1-24
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Y	<p>RUANO G ET AL: "BOOSTER PCR: A BIPHASIC PARADIGM FOR AMPLIFICATION OF A FEW MOLECULES OF TARGET" AMPLIFICATIONS: A FORUM FOR PCR USERS, PERKIN-ELMER CO., NORWALK, CT, US, 1 September 1989 (1989-09-01), pages 12-13, XP000567442 the whole document</p> <p>---</p>	1-24
Y	<p>PICARD C ET AL: "DETECTION AND ENUMERATION OF BACTERIA IN SOIL BY DIRECT DNA EXTRACTION AND POLYMERASE CHAIN REACTION" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 58, no. 9, September 1992 (1992-09), pages 2717-2722, XP000946427 ISSN: 0099-2240 abstract page 2718, left-hand column, paragraph 3 page 2719, right-hand column, paragraph 2</p> <p>---</p>	1-24
Y	<p>SAULNIER P ET AL: "DETECTION OF GENES IN FECES BY BOOSTER POLYMERASE CHAIN REACTION" JOURNAL OF CLINICAL MICROBIOLOGY, WASHINGTON, DC, US, vol. 30, no. 8, August 1992 (1992-08), pages 2080-2083, XP001084737 ISSN: 0095-1137 abstract page 2081, left-hand column, paragraph 2</p> <p>---</p>	1-24
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>WO 97 19191 A (OEGREID DAGFINN ;ULVIK ARVE (NO); KOORNSTRA JAN JACOB (NL)) 29 May 1997 (1997-05-29) the whole document, in particular page 3, lines 26-29</p> <p>---</p>	1-24
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